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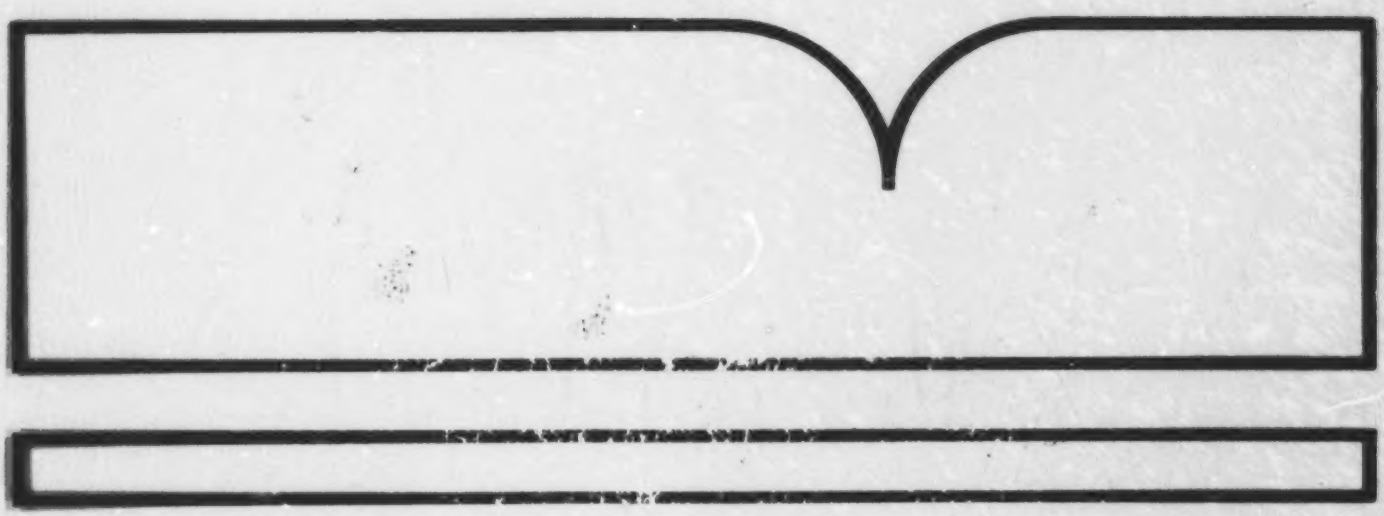

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16. ABSTRACT

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A complete handbook of basic laboratory procedures, replete with resource information of use to environmental microbiologists for practical applications and research was published. Sections on growth measurement discuss direct, colony, and most probable number (MPN) counts, cultural, enrichment, and selective media, isolation techniques, and related statistics. Physical methods include photometry, chromatography, radioactivity, and other techniques applicable to rapid measurements and automation. Diluents, biomass measurements, and laboratory safety are also addressed.

A large volume on prokaryotes provides practical source material on cultural, enrichment, and selective techniques of interest to aquatic microbiologists. Chapters on Enterobacteriaceae, indicators, pathogens, and marine bacteria describe isolation and characterization methods for each species, including those important to water quality. A comprehensive text on membrane filtration contains chapters on the structure, characteristics, and standardization of membrane filters (MF), and reviews MF methods for the examination of water.

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Water Pollution

Microbiology: methodology and quality assurance

Robert H. Bordner

METHODS

Research on the microbiological quality of water continues to concentrate on improved methods for the detection, enumeration, and identification of pollution indicators, pathogens, and other microbial groups.

A complete handbook of basic laboratory procedures, replete with resource information of use to environmental microbiologists for practical applications and research was published.[1] Sections on growth measurement discuss direct, colony, and most probable number (MPN) counts, cultural, enrichment, and selective media, isolation techniques, and related statistics. Physical methods include photometry, chromatography, radioactivity, and other techniques applicable to rapid measurements and automation. Diluents, biomass measurements, and laboratory safety are also addressed.

A large volume on prokaryotes provides practical source material on cultural, enrichment, and selective techniques of interest to aquatic microbiologists.[2] Chapters on the *Enterobacteriaceae*, indicators, pathogens, and marine bacteria describe isolation and characterization methods for each species, including those important to water quality. A comprehensive text on membrane filtration contains chapters on the structure, characteristics, and standardization of membrane filters (MF), and reviews MF methods for the examination of water.[3]

ISOLATION AND ENUMERATION PROCEDURES

In a comparison of MF and MPN methods for testing drinking water, Hsu and Williams[4] found that MF recovered more

coliforms with m-Endo LES agar than the MPN procedure, although some nonsheen colonies commonly verified as coliforms. Verification by parallel inoculation into lauryl tryptose (LT) and brilliant green bile broth (BG) was more efficient than the recommended LT enrichment before transfer to BG. Growth from confluent plates was inoculated directly into BG broth for coliform recovery. The authors recognized that overgrowth on some MFs was a result of prolonged sample holding time. On the other hand, they suggested that evaluation of routine test data and bacterial reductions over time indicated that non-quantitative recovery of coliforms may not be significantly affected by holding samples up to 72 hours. Standridge and Delfino[5] found that some coliforms in the total coliform MF test, primarily *Citrobacter freundii,* produced gas from lactose in BG, but not in LT, and that the negative results were related to the peptones in the media. McFeters *et al.*[6] used stressed cultures of *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae,* and *C. freundii,* and nonstressed controls to study the effects of diluent composition, temperature, exposure time, various formulations of media, and MFs of different pore sizes in the performance of the total coliform test. Peptone or milk solids added to diluents and held at 4°C maximized recovery of injured cells, but had little effect on the controls. Most of the media commonly used in water analyses recovered less than 30% of the injured cells, but control cells were recovered well on most media tested. Injured bacteria were sensitive to more than 0.01% deoxycholate in these media. MF surface pore morphology had little effect on total coliform recoveries. Raw and treated drinking water samples were analyzed using the MF and presence-absence (P-A) tests.[7] Coliform isolates were identified to species from 15 130 samples collected over a 2-year period. These data were examined according to category to determine frequency of isolation, influence of water source (well, lake, river, or mixed), and effectiveness of treatment. The species that occurred most often among all water types were *E. coli, E. aerogenes, K. pneumoniae, C. freundii,* and *Aeromonas hydrophila.* Modified MacConkey broth and lactose lauryl tryptose tryptone broth (LLTT) were compared as presumptive media in the P-A test. Because the differences in recovery were not significant, LLTT was recommended as a presumptive coliform medium. This medium also detected Gram-positive organisms of sanitary significance. The P-A test was proposed as a simple, reliable method for analyzing and monitoring drinking water quality more frequently than presently required under drinking water regulations.

For fecal coliform enumeration, Pagel *et al.*[8] compared four MF media for accuracy, specificity, upper counting limit, and recovery comparability. Accuracy, as determined with pure cultures, ranged from 89 to 100% for m-FC, mTEC (originally designed to enumerate *E. coli*), and m-FC2 (2 hours, 35°C resuscitation period), but was less than 60% for MacConkey broth. These values decreased approximately 40 to 55% when the cultures were stressed at 10°C. Specificity was determined by verification of almost 800 colonies. False positives occurred most frequently (18%) with the m-FC2 method, and false negatives with MacConkey broth (26%). mTEC achieved the highest upper counting limit with 130 colonies per filter. In general, recoveries from over 130 natural samples of various types were significantly higher with the m-FC2 and mTEC methods. However, growth of background organisms was also higher on mTEC medium. The mTEC method provided the highest recovery from chlorinated effluents, and the addition of a 2-hour, 35°C resuscitation period to this medium improved recovery even more. The best overall fecal coliform enumeration was obtained by the mTEC method, which also had the advantage of shorter (24-hour) incubation. Similarly, Cabelli *et al.*[9] showed that the mTEC method yielded significantly higher fecal coliform densities in estuarine and coastal water samples than the standard method and the preincubation (2 hours at 35°C) modified m-FC procedure. The mTEC colony verification frequency exceeded 95%. Patrick and Kendrick[10] found that more than 7 mg of fine suspended solids (<200 μm) deposited on an MF affected coliform colony growth and integrity and resulted in uncountable membranes. A smaller amount of finer sediment (<44 μm) produced the same effect. When MFs were countable, no correlation between suspended solids concentration and ratio of coliform MF to MPN was obtained.

In a comparative evaluation of various methods for detecting coliforms, fecal coliforms, and *E. coli* in water, Stelzer *et al.*[11] concluded that lactose-base tube media offered improved coliform recovery over MF methods because the latter required verification with the cytochrome oxidase test and test sensitivity was more limited. The coliform tests were incubated at 35° to 37°C and the fecal coliform analyses at 44.5°C. Traditional confirmatory tests were carried out in liquid or on plate media and included the IMViC tests for *E. coli.* Although standardization is desirable, the author concluded that frequency of testing was more important than the actual test method in determining water quality. Austin *et al.*[12] used numerical taxonomic procedures to determine the range of bacterial taxa isolated from fecal and completed total coliform MPN analyses of oysters, water, and sediments. As expected, they found a diverse range of bacteria, some of which lost the ability to ferment lactose aerogenically (although stored on lactose-containing media), and others that were of little sanitary significance. The large number of non-*Enterobacteriaceae* isolates and the poor recovery of injured cells supported the contention of some workers that the coliform count served as a tenuous measure of unsanitary conditions, and that improved tests for water quality were needed. Identification of isolates by the API-20E system gave 83% agreement with conventional methods. The discrepancies occurred with *C. freundii, Erwinia herbicola, Serratia liquifaciens,* and "unidentified Gram-negative rods." These results supported the suggestion that commercially available kits, such as API 20E, acceptably identify commonly occurring, well-defined members of the family *Enterobacteriaceae* from clinical specimens, but do not yield reliable results in the identification of atypical strains commonly isolated from the aquatic environment.

A comparison of confirmatory media for coliforms and *E. coli* in water determined that LT and BG were both satisfactory for gas production at 37°C, but LT gave fewer false negative results and was therefore significantly less inhibitory than BG at 44°C.[13] However, when LT was compared with lauryl tryptose mannitol broth (LTM), with and without tryptophan for both acid and gas production, LT gave a higher proportion of false negative reactions in the indole test at 44°C. Substitution of mannitol for lactose and addition of tryptophan yielded a satisfactory medium for confirmation of gas production and for indole demonstration at 44°C. This was approved by the

Public Health Laboratory Service and the Standing Committee of Analysts (PHLS/SCA) of Great Britain. Smith and Rockliff[14] proposed a rapid single-tube confirmatory test for *E. coli* that used a modification of the PHLS/SCA-approved medium. The modified LTM broth was used in 0.1-ml volumes and incubated 4 hours at 44°C. It provided 99% agreement with traditional methods. Parker[15] reported that a modified fecal coliform MF medium (m-FC) improved resolution and accuracy for the enumeration of bacteriophage MS2 plaques in a drop-count technique. A selective MF medium was described for rapid enumeration of *E. coli* in tropical waters, where the numbers of fecal coliforms other than *E. coli* yielded inaccurate results.[16] The medium gave MF results comparable to m-FC medium and membrane-enriched Teepol broth, and to MPN values when using Gray's glutamate broth. Feng and Hartman[17] developed a rapid method for confirming the presence of *E. coli* in MPN tubes by incorporating 4-methylumbelliferome glucuronide (MUG) into LT broth at a final concentration of 100 μg/ml. MUG was hydrolyzed by glucuronidase to produce a fluorogenic compound, and only strains of *E. coli* and some *Salmonella* and *Shigella* strains in the family *Enterobacteriaceae* produced glucuronidase. Results from the presumptive test (gas production) and the confirmed test (fluorescence) for *E. coli* were obtained after 24 hours of incubation at 35°C. Approximately 90% of the tubes with these reactions were positive in EC broth incubated at 45°C. Few false positive reactions occurred. A rapid confirmatory test for *E. coli* adaptable to automation was developed by filling microtitration plates with a nonselective medium containing MUG. Pure or mixed cultures containing *E. coli* fluoresced within 4 hours (most strains) to 24 hours (a few weakly positive strains). Enterotoxigenic *E. coli* were detected in drinking and bathing water samples by using a recombinant DNA hybridization test for genes encoded for *E. coli* enterotoxins.[18] Water samples were filtered and bacterial cells on the MF were processed and examined with alpha-^{32}P-labeled DNA probes to detect the presence of specific enterotoxins. The procedure was a much more sensitive and less cumbersome means of detecting organisms carrying genes coded for enterotoxin production than was testing 10 individual colonies in tissue culture and suckling mice assays.

Investigations by Althaus *et al.*[19] compared selective media for fecal streptococci and found bile aesculin agar and gentamicinthallous carbonate agar to be less sensitive than citrate-azide-tween-carbonate agar, KF streptococcal agar, enterococcal agar, and Kanamycin aesculin azide agar. The latter performed slightly better than the other media for the analysis of drinking water. No significant differences were found in the end concentrations of glucose, which ranged from 0.5 to 1.0%, and of sodium azide, which ranged from 0.02 to 0.05%. Incubation at 36°C provided better recovery than at 44°C, and results were better after 24 hours than after 48 hours. Havelaar *et al.*[20] compared four media for confirming the presence of Group D streptococci from azide dextrose broth enrichment cultures inoculated with water samples and incubated at 37°C. Ethyl violet azide broth incubated at 37°C did not support growth of all Group D streptococci tested, frequently failed to produce the typical "purple button," and gave positive reactions with some non-Group D strains. Azide dextrose broth incubated at 44°C was also inhibitory to some Group D streptococci and was sometimes overgrown by catalase-positive bacteria. Bile esculin azide agar (BEA) at 44°C supported growth of Group D streptococci almost exclusively, but was the most inhibitory. KF streptococcus agar at 37°C gave positive reactions with most Group D streptococci, but also with non-Group D strains. Based on these results, BEA and KF agars were recommended.

The effects of the insecticide carbaryl on *E. coli, Streptococcus faecalis, Salmonella typhimurium,* and *Staphylococcus aureus* were tested using plate counts and Warburg respirometry.[21] Gram-negative *E. coli* and *S. typhimurium* were initially stimulated and Gram-positive *S. faecalis* and *S. aureus* were inhibited. But, over a period of 8 days, all four organisms died off more rapidly in the presence of carbaryl than in the controls. Thus, small amounts of carbaryl in the water could have caused invalid interpretation of bacteriological data on water quality. The indicator, *S. faecalis,* died off faster than the pathogen, *S. typhimurium,* and both indicators simulated aftergrowth by initially increasing.

The pour-plate was compared to the spread-plate method for standard plate count (SPC) on samples collected from a water distribution system during routine flushing of dead-end mains.[22] A statistical analysis of the results demonstrated that the pour-plate was neither as accurate or as precise as was the spread-plate procedure for enumerating the heterotrophic (SPC) plate count population. Haas *et al.*[23] compared the SPC with a membrane filter SPC procedure (m-SPC) on water samples from two New England water treatment plants. Computerized analyses of the data indicated that the m-SPC was a satisfactory alternative to the SPC method, and was more sensitive because it could accommodate sample volumes greater than 1.0 mL.

A technique was reported for enumerating bacteria in river water and wastewater effluent in which the medium was spread-inoculated with calibrated loops delivering 0.01 or 0.001 mL.[24] A comparison of the loop-plate method with the standard spread-plate method yielded no significant differences, but the loop method provided a simple, rapid, reliable means of inoculating agar media for estimated bacterial populations in water. A pour-plate method using 10 plates at each of 2 dilutions was used to determine the aerobic plate count of bacteria in tropical surface water.[25] The plates were incubated at 20°, 25°, 30°, 35°, and 40°C, and counted after 24, 48, 72, 96, and 120 hours. After statistical analysis of the results, incubation at 30°C for 72 hours was recommended as a standard procedure for aerobic plate counts in warm tropical waters. Membrane filtration and epifluorescent microscopy were used for rapid enumeration of bacteria in rinses of milking equipment.[26] Rinse water was filtered through a 0.6-μm pore size Nuclepore MF and the retained bacteria were stained with acridine orange. The counts of orange fluorescing bacteria correlated well ($r = 0.83$) with corresponding plate counts. The technique was rapid (approximately 10 minutes), sufficiently sensitive to detect 1000 bacteria/mL, and distinguished actively growing from nonviable bacteria. Denitrifying bacteria were counted using a PY-Nitrate liquid medium with the MPN technique.[27] The ratios of denitrifying bacteria to aerobic heterotrophic bacteria were determined, and samples from a river and various depths of a stratified, eutrophic lake and a non-stratified eutrophic pond and lake were analyzed. Almost all denitrifying bacteria were Gram-negative. *Pseudomonas* was the genus most frequently isolated. Suzuki *et al.*[28] reported on

a MF method for isolating and characterizing thermophilic bacteria incubated at 70°C that provided improved colony formation and recovery. They theorized that the MF method retained surface moisture and held down motility better than agar surfaces.

Many researchers have developed methods for waterborne pathogens of recent interest. Chan and Mackenzie[29] reported that enrichment with a semi-solid medium increased the isolation rate of *Campylobacter fetus* subsp. *jejuni* and also served as a successful transport medium. They also suggested that a useful control system was to streak *Pseudomonas aeruginosa, Clostridium perfringens,* and a laboratory strain of *Campylobacter* on a blood-agar plate and incubate in a microaerophilic atmosphere. Good growth of all three organisms indicated satisfactory culturing conditions for *Campylobacter.* Moskowitz and Chester[30] tested a total of 67 oxidase-positive, Gram-negative bacteria for growth on selective *Campylobacter* agar (Blaser formulation) at 42°C under microaerophilic conditions. Most of the cultures did not grow, but all strains of *Achromobacter xylosoxidans, Ps. aeruginosa, Ps. putrefaciens, Ps. alcaligenes,* and *Ps. pseudoalcaligenes* did grow on the medium. An improved selective medium for isolating *Campylobacter* from fecal and environmental samples was developed by Bolton and Robertson.[31] Addition of polymixin, rifampicin, trimethoprim, and actidione provided the improved selectivity. Ribeiro *et al.*[32] also proposed an improved medium for the isolation and purification of *Campylobacter* species from feces and water samples. *Campylobacter* species were also grown in a medium developed by Mehlman and Romero[33] and maintained for 1 year with transfer every 2 weeks. The base medium contained proteose peptone No. 3, yeast extract, K_2HPO_4, $(NH_4)_2SO_4$, Na_2SO_3, soluble starch, and agar. Concentrations and sources of organic nitrogen and growth factors were critical. The optimal pH range was 7.0 to 7.5, and cultures were sensitive to surface-active agents at concentrations used for enrichment of other Gram-negative bacteria. Bolton *et al.*[34] described an MPN procedure for *Campylobacter fetus* that successfully enumerated the organisms in coastal, estuarine, and fresh water samples and estimated as few as 10 per 100 mL. For primary isolation of *Campylobacter fetus* from samples, Luechtefeld *et al.*[35] found that the conventional incubation atmosphere (5% oxygen and 8% carbon dioxide) was superior to the candle jar (17% oxygen and 3% carbon dioxide). At 42°C, the 5% oxygen atmosphere produced consistently larger *Campylobacter* colonies and more colonies per plate.

To recover *Clostridium perfringens* spores from sediment samples, Emerson and Cabelli[36] used extraction-separation procedures in conjunction with mCP medium and the MF. The precision of the sonication and settling method was markedly better than the results theoretically obtained by the MPN method.

Direct plating methods using antimicrobial agents in the media were significantly more sensitive for isolating *Legionella pneumophila* from potable water specimens that guinea pig inoculation. Edelstein *et al.*[37] analyzed 85 samples and found 14 positive for *Legionella* by the plating method, and 2 by guinea pig inoculation only. The same group tested 18 types of proposed media for *Legionella,* all of which used charcoal yeast extract and 15 of which contained antimicrobial agents.[38] A medium containing cefamandole, polymyxi B, anisomycin, alpha-ketoglutarate, and an organic buffer (BMPA alpha) in conjunction with an acid wash treatment, recovered best from clinical specimens and potable water. Johnson *et al.*[39] formulated a simple medium for *Legionella* containing yeast extract, phosphate, and hemin as the major components. The medium supported a wide range of clinical and environmental isolates, and plating efficiencies were comparable to those of charcoal-yeast extract agar. The medium should be suitable for genetic and antibiotic susceptibility studies, skin tests, and production of biological reagents, because it is buffered and transparent. Using another approach, Janssen and Hedlund[40] proposed using a filtered yeast extract agar containing polyvalent antiserum for the detection and direct isolation of *Legionella* species.

A comparative study of three media for the isolation of *L. pneumophila* was made on 100 water samples.[41] Buffered charcoal-yeast extract medium (BCYE) with an acid wash detected 83% of the 24 positive samples. BCYE medium with antimicrobial agents (BMPA) detected 79%, and BCYE medium with antimicrobial agents, glycine, and differential dyes (MWY) detected 92%.

Wakisaka and Koizumi[42] devised a selective medium for the isolation of *Pseudomonas* bacteria containing an antibiotic mixture of cerexin A, nalidixic acid, and cycloheximide. *Pseudomonas* species were isolated from samples containing about 400 times as many other Gram-positive and -negative bacteria, and 58 strains of *Pseudomonas* were identified.

Selenite, tetrathionate, and Rappaport's media were compared for *Salmonella* isolation after pre-enrichment in buffered peptone water at 37°C for 18 hours and subculture to the three media.[43] Tetrathionate and selenite were incubated at 43°C for 48 hours, and Rappaport's medium was incubated at 37°C for 48 hours. Subcultures from the three enrichment broths were made at 24 and 48 hours to brilliant green MacConkey agar and incubated at 37°C for 24 hours. Rappaport's medium was significantly more efficient than the other two for *Salmonella* recovery. These results from food samples reinforced previous findings from wastewater polluted water. It was suggested that Rappaport's medium alone be used for the routine examination of environmental samples. A later comparison evaluated the R25 modification of Rappaport's enrichment medium incubated at 37°C and strontium chloride B enrichment at 37° and 43°C.[44] Elevated temperature enrichment improved the performance of strontium chloride B, but Rappaport's medium still gave significantly better results. This supported earlier studies on the simplification and standardization of enrichment media. Rappaport broth (25) incubated at 37°C was recommended as the medium of choice.

In a comparative evaluation of selective media for isolation of salmonellae and shigellae from fecal samples, Goyal *et al.*[45] cultured 113 samples on eosin-methylene blue, brilliant green, Hektoen (HEK), xylose-lysine desoxycholate (XLD), MacConkey (MAC), salmonellae-shigellae (SS), bismuth sulphite (BS), and desoxycholate-citrate (DC) agars. Salmonellae were isolated from 32.7% and shigellae from 10.6% of the samples. XLD, HEK, MAC, and BG agars gave the best isolation of salmonellae. XLD and MAC agars were recommended for routine analyses of fecal samples for salmonellae and shigellae.

McTighe[46] reviewed the occurrence, microbiological and serological characteristics, culture techniques, selective media, and identification methods for pathogenic *Vibrio* species. Thiosulfate-citrate-bile salt (TCBS) agar was recommended for

the isolation of the halophilic vibrios from clinical and environmental samples, and growth reactions of other bacteria that may grow on this medium were described. A simplified procedure for identification of the isolates was described.

A thorough review of the occurrence, taxonomy, epidemiology, clinical symptoms, and isolation and identification procedures for *Yersinia enterocolitica* was given by Swaminathan *et al.*[47] Water and other environmental sources were described. A comparative study of seven selective differential media for the isolation of *Y. enterocolitica* indicated that cefsulodin-irgasan-novobiocin (CIN) and BS agars were most efficient. MAC and MAC with Tween 80 agars were acceptable, and cellobiose-arginine-lysine (CAL), SS, and DC agars were unsatisfactory for isolation from artificially inoculated pork. Differentiation of *Yersinia* species by biochemical reactions was described. Bartley *et al.*[48] developed a MF procedure for the isolation of *Y. enterocolitica* from aquatic environments, based on the fermentation of sorbitol, the production of urease, and the absence of lysine decarboxylase and arginine decarboxylase-dihydrolase activities. Presumptive identification of *Y. enterocolitica* was accomplished in 50 hours and the confirmation rate of typical colonies was 88%. Another comparative study of selective media for recovery of *Y. enterocolitica* evaluated six proposed media using strains most commonly associated with human disease in the U. S. and Canada, and with randomly picked stool specimens.[49] The combined recoveries of the pure cultures on test media as compared to blood agar were MAC—75%; SS—48%; pectin—70%; CAL—62%; Y medium—15%; and CIN—85%. The study found CIN medium to be by far the most effective. It was highly selective and almost completely inhibited the fecal flora.

Butcher and von Groevenitz[50] evaluated dextrin-fuchsin-sulfite medium (DFS), Rimler-Shotts agar (RS), and a new lysine-ornithine-mannitol agar (LOM) for detection of *Enterobacter agglomerans*. LOM and DFS were most sensitive to coliform/*E. agglomerans* ratios of $\leq 10^2$ and *E. agglomerans* inocula of $>10^2$ per plate, but RS was inhibitory.

A new medium (FRAG agar) for the selective isolation and presumptive identification of *Bacteroides fragilis* was developed and evaluated by Lyznicki *et al.*[51] The medium contained 1% D-glucuronic acid as a fermentable carbon source, reduced peptone content, gentamycin, and 20% bile. Presumptive identification was based on growth, fermentation, and typical colony morphology. A total of 75 stock culture isolates of the *B. fragilis* group grew well on FRAG agar and 69 showed evidence of fermentation and typical colony morphology. Of 90 other anaerobes, none grew well or fermentated glycuronic acid. In a clinical trial of 100 specimens cultured anaerobically, 33 isolates were recovered on the selective medium, but only 23 were detected by routine methods.

A MF method for the quantitative recovery of *Acinebacter calcoaceticus* from water was developed and evaluated by Lacroix and Cabelli.[52] The medium used a mineral base, sodium acetate (carbon source), potassium nitrate (nitrogen source), and formic acid to inhibit background growth. The test was incubated for 46 hours at 30°C, which was followed by fermentation and cytochrome oxidase checks on the colonies as they appeared on the MF. The confirmation rate of presumptive *A. calcoaceticus* colonies was 95%, whereas 8% of the negative colonies were *A. calcoaceticus*. The precision of the method did not exceed that expected from random error alone. Background microbial growth decreased an average 1.77 orders of magnitude.

Koburger and May[53] evaluated Bennett, Ryalle and Moss, and *Aeromonas* MF agars for their ability to recover *Chromobacterium* species from foods, soil, and water samples. The tests were incubated at 25 and 35°C. Bennett agar recovered best both *C. violaceum* and *C. lividum*, a psychrotroph, at 25°C. *Aeromonas* agar at 35°C gave the highest recoveries of *C. violaceum*.

A useful review of iron bacteria, their occurrence and economic significance, and procedures for their isolation, enumeration, and identification, was given by Smith.[54] Methods were described for the rapid and reliable identification of *Gallionella ferruginea* and species of the *Leptothrix* and *Sphaerolilis* groups isolated from water.

Fiksdal *et al.*[55] reported on various non-standard enumeration procedures for determining maximum viable counts of bacteria in drinking water. They included five MF media, incubation at 20°C, and incubation up to 14 days or longer. Differences in the results from various drinking water sources and from those of other investigators indicated that regional variations in bacterial flora may have explained the differences in recovery by the various enumeration procedures. Strain diversity was also estimated for heterotrophic bacteria from chlorinated and nonchlorinated waters. Untreated waters showed much greater diversity than chlorinated samples.

An improved method for determining respiring microorganisms in natural waters was reported by Tabor and Neihof.[56] The method combined microscopic determination of specific, individual respiring organisms by the detection of electron transport systems activity after staining with acridine orange, and the total number of organisms by epifluorescence microscopy. A comparison was made between acridine orange (AO) and 4,6-diamidino-2-phenylindole (DAPI), a highly sensitive DNA fluorescing stain, for enumerating aquatic bacteria.[57] No significant difference was observed between populations estimated with AO and 0.05 μm/mL DAPI. However, bacteria were more easily distinguished from other particulate matter with DAPI. Acridine orange direct counts were used to study the bacteriology and heterotrophic activity of a stream and nearby groundwater.[58] Indexes of metabolic activity were also measured by testing the use of labile dissolved organic matter with glutamic acid, phenylalanine, and glycolic acid as substrates. Bacterial populations in the groundwater were greater than in the stream and the metabolic activity indexes were higher.

In a statistical analysis of the direct count method, Kirchman *et al.*[59] showed that most variance in the method (less than 80%) was caused by microscope fields, and the rest was caused by filters. The optimal strategy for providing the lowest possible variance at a given cost was to prepare one filter from two or preferably three subsamples using the greatest replication possible at each performance level. Rapid enumeration of viable bacteria in marine sediment was accomplished using an epifluorescent microscopic method after incubation with nalidixic acid.[60] This technique permitted easy recognition of the viable cells, and *in situ* observations of the viable microorganisms attached to sand grains. A comparison with the classic agar plate showed the superiority of this technique.

Buck[61] described a rapid KOH method for determining the Gram reactions of bacteria as applied to marine isolates. All

positive and negative results obtained by Gram staining were confirmed by the KOH method. Gram-variable bacteria gave equivocal results. Lysis by KOH and hydrolysis of L-alanine-4-nitro-anilide (LANA) were compared with the Gram reaction of aerobic, microaerophilic, and anaerobic bacteria.[62] Both tests correlated well with the Gram reaction for nonfermentative bacilli and *Bacillus* species. However, they did not correlate with nonsporulating anaerobes. Only campylobacters were KOH-positive and LANA and Gram-negative.

IDENTIFICATION SYSTEMS

Advances in single and multitest systems for identification of microorganisms were frequently reported. Matson and Bale[63] evaluated a rapid kit method (Micro-ID), an overnight incubation kit method (API 20E), and conventional biochemical tests to compare laboratory effort, time, and cost. A total of 730 *Enterobacteriaceae* strains were evaluated, and 64% were processed by the Micro-ID within 24 hours. An additional day was required by the API and conventional methods. The Micro-ID also required less time for set-up and interpretation (4.5 minutes) than the API (6 minutes) or conventional method (7 minutes). Total direct costs per organism were: Micro-ID—$4.30; API 20E—$4.96; and conventional biochemicals with commercially prepared media—$5.66. To adapt the API 20E system to the characterization of marine and estuarine bacteria, McDowell *et al.*[64] used a diluent composed of 20% marine salts and incubated at 22°C. They were successful in rapidly characterizing halophilic strains normally nonreactive by the API 20E system.

Enteric-Tek (ET), a new system for the identification of *Enterobacteraccae*, is a round, multi-compartmented plastic plate with 11 peripheral wells and 1 center well incorporating 14 biochemical reactions. One reagent (Kovacs) is required to complete the biochemical tests and to generate a five-digit number for computer code identification. Bruckner *et al.*[65] compared the ET wheel to conventional tests and the API 20E, and found it to be convenient, simple, rapid, and highly reliable. The ET and Auto Microbic system (AMS) were compared to the conventional biochemical tests to identify 201 *Enterobacteriaceae*.[66] All tests were inoculated simultaneously from the same MacConkey plate. Identifications made with the ET and AMS agreed with those from conventional tests 97% of the time. The AMS and ET identified the clinical isolates with comparable accuracy. However, the AMS provided a significant time savings. The ET identified 75% of the isolates within 18 hours without the aid of additional biochemical tests, and the AMS identified 92% in 8 hours. Esaias[67] evaluated the ET wheel and compared it with conventional methods for identifying 301 enteric cultures representing 36 species. The ET system correctly identified 97.8% of the 270 typical strains, and 83.9% of the 31 atypical strains, giving an overall accuracy rate of 96.3%. The ET system was reliable and accurate at the species and genus levels within 18 to 24 hours. The AMS has recently made available, in addition to the Enterobacterioaceae Biochemical Cards (EBC), modified cards (EBC+) to identify certain nonenteric bacilli. Minor modifications were made in the computer program and in the interpretation of test results with the new cards. The nonenteric organisms were *Ps. aeruginosa, Ps. fluorescens, Ps. putida, Ps. maltophilia, P. cepacia,* and *Acinetobacter calcoaceticus*. Smith *et al.*[68] examined 410 such glucose-nonfermenting Gram-negative rods to test the capability of the EBC+, which correctly identified 366 (89.3%). The EBC and EBC+ cards were tested in parallel and found to be in agreement with 97% of 650 *Enterobacteriaceae*, after equivocal AMS results were excluded.[69] Equivocal responses occurred with 4% of EBC and 7% of EBC+ tests. A total of 159 nonenteric clinical isolates were identified by Johnson and Brinkley[70] using the EBC+ and conventional tube biochemicals, based on an established identification scheme. The AMS EBC+ correctly identified 96.7% of isolates for which taxa were included in the data base. AMS offered automated identification of frequently isolated nonenterics in 8 to 13 hours. When compared with conventional media, the N/F system and the OxiFerm tube correctly identified 97.7% of infrequently encountered nonfermentative Gram-negative bacilli from clinical isolates.[71]

A rapid carbohydrate oxidation microtube system designed for detecting the saccharolytic activity of Gram-negative, nonfermenting bacilli, was evaluated and compared to the conventional oxidation-fermentation method.[72] More than 95% of isolates gave correct oxidation reactions within 4 hours in the microtubes, whereas standard oxidation-fermentation media required 24 hours. The oxidation of glucose, maltose, lactose, and xylose was tested with 430 strains of *Pseudomonas, Acinetobacter, Achromobacter, Alcaligenes, Moraxella, Flavobacterium,* and *Bordetella* species. The microtube system was simple, accurate, rapid, and convenient.

Mugg and Hill[73] compared two new miniaturized identification systems, the Microbact-24E (M-24E) and the Microbact-12E (M-12E) to the API 20E for the identification of 352 fresh clinical isolates of *Enterobacteriaceae*. The M-24E identified 98%, API 20E identified 94.3%, and the M-13E identified 88.6%. All three systems were easy to use, and were provided with computerized profile registers to assist in final identifications. The M-24E system was found to be a very accurate and convenient identification system.

Using a variety of sporeforming and non-sporeforming anaerobic bacteria, Lombard *et al.*[74] compared the Anaerobe-Tek (A/T) system recently marketed and the Presumpto-Plate systems with conventional procedures. Agreement of 16 basic characteristics was from 92.8 to 100%. Of the 204 strains of common anaerobes tested with the A/T system, only 70% were correctly identified to the species level. However, several additional strains could have been identified if certain other characteristics were included in the A/T data base.

A kit for the identification of staphylococci was used to identify 100 strains of coagulase-negative staphylococci isolated from various clinical isolates.[75] Over 90% of the staphylococci were easily identified to species according to their reaction profile to 19 bio-chemical tests included in the kit. The remainder, which showed minor variations, were also assigned to species. A new microtube method for the detection of coagulase production by *Staphylococcus aureus* was evaluated and no false-positive or false-negative reactions resulted.[76] The data showed that 99% of the coagulase-positive strains tested could be detected within 4 hours by the microtube method, which was convenient and economical.

A new commercially available latex agglutination test was compared to the tube coagulase and slide coagulase tests for identifying *S. aureus*.[77] Of 160 isolates, 99.4% gave positive

results. The test was found to be rapid, simple, as accurate as the tube coagulase test, and more accurate than the slide coagulase test.

Using biochemical characteristics determined by conventional methods and multitest systems, Burke *et al.*[78] classified 93% of *Aeromonas* biotypes correctly according to enterotoxin production in a study of 174 strains. Most enterotoxigenic strains were Voges-Proskauer (VP) positive and did not hydrolyze arabinose. VP-positive strains that did hydrolyze arabinose were nonenterotoxigenic.

Strains of *Clostridium perfringens* and culturally similar species were examined by using conventional confirmatory tests, the API ZYM system, and individual tests for phosphatase and glutamic acid decarboxylase (GAD) activity.[79] The API ZYM incorporated 19 different enzymes and distinguished *Cl. perfringens* from several other *Clostridia.* A single test for acid phosphatase helped distinguish the closely related species, but GAD was not useful.

Filter paper disks containing defined amounts of bile salt were placed on a lawn of suspected *Bacteroides* cells in Gifu anaerobic medium agar plates and incubated in anaerobic jars containing hydrogen and carbon dioxide generators at 35°C for 24 hours.[80] Bile-resistant bacteria were identified as *Bacteroides fragilis.* Four reagents, Kovacs, p-dimethylaminobenzaldehyde, Ehrlich, and p-dimethylaminocinnamaldehyde (PDAC), were compared in spot indole tests on 359 strains of Gram-negative rods growing on 5% sheep blood agar, trypticase soy agar, and MacConkey agar.[81] the PDAC reagent was the most sensitive, and provided results easiest to interpret. Schindler and Idlbek[82] developed a simplified strategy for the identification of Gram-negative fermenting rods using a desktop computer. They used a matrix of 54 taxa and 29 tests in identifying correctly 98% of 845 strains. A sheet for optical page marks was developed by Dott and Trampisch[83] to transfer the large amount of identification criteria necessary for quantitative and qualitative determinations of bacterial populations to the computer-assisted analysis. Cluster analysis and formal descriptions were used to classify bacteria.

Deininger[84] described a water quality data bank system that allowed the user to direct-search, update, retrieve, and analyze data from a computer terminal. Almost all statistical operations could be performed on the data, including histograms, distributions, correlations to regression, and component and spectral analyses. Commands for producing camera-ready copy were available. The system was activated on the University of Michigan Computer System and accessed through local telephone numbers in more than 100 cities in the U. S. and Canada. The operation of the system was illustrated on a small data base on Ohio River water quality provided by the Ohio River Sanitation Commission.

Lefevre and Gavini[85] described the theory and programming of a computer system to identify fecal coliforms and newly described aquatic species. In applying this system, Gavani *et al.*[86] tested 279 enteric bacteria isolated from clinical material and 564 strains isolated from drinking water samples. The results compared favorably with the API 20E and Micro ID methods. Fecal coliforms and new species of aquatic origin were characterized by numerical analyses and DNA-DNA hybridization procedures.

Koch[87] developed a computer MPN test and expanded the index tables to handle greater numbers of culture tubes than the five-tube MPN. The program was written for a hand-held, advanced programmable pocket calculator.

RAPID METHODS

A rapid method for determining quality in natural water systems by enumerating coliphages was reported by Wentsel *et al.*[88] Correlations were established between coliphages and coliforms, which were used to determine the number of total and fecal coliforms present. In waters with coliphage concentrations ≥6/100 mL, coliphages were enumerated within 6 hours. The technique was evaluated in water laboratories throughout the U. S.

Drucker[89] reviewed the applications of gas chromatography to microbiology, including gas-liquid chromatography, analysis of fermentation products, and pyrolysis-gas chromatography. Tilton[90] edited a review covering the status of rapid methods and automation in microbiology. The applications of automation to the rapid detection of microorganisms in fluids, foods, environmental, and clinical samples were addressed. The Autobac, MS-2, and other systems were discussed, and nonautomatic rapid methods were described.

QUALITY ASSURANCE

The importance of establishing a quality assurance (QA) program for biological environmental studies was discussed by Reed.[91] He pointed out that data from some recent studies have been questioned, and that means for evaluating precision, accuracy, and reliability of data, and adequate documentation are necessary. The elements of a QA program were described. The operation of a comprehensive proficiency sample program for all clinical microbiology laboratories in the U. K. was described by Snell *et al.*[92] Water and milk samples were included among the wide variety of samples used. Participating laboratories were requested to examine the specimens using their routine procedures, and report the results to the microbiological quality control laboratory, where the reported values were analyzed. Summarized results of each distribution were sent to all participants. Laboratory performance was reviewed semiannually, and laboratories with results significantly worse than those of their peers were offered technical assistance from a national advisory panel. Feldman[93] pointed out that as microbiologists develop and modify methods, and turn to automated procedures, the precision and accuracy of the methods must be ensured through collaborative study. He cited sterility testing and biological indicators as QA subjects of high interest to the U. S. Food and Drug Administration, and to industry. The cost effectiveness of quality control (QC) required by regulators was studied, and surveillance procedures were evaluated according to the frequencies of deficiencies that resulted over 15 years in a clinical microbiology laboratory.[94] Potential savings from elimination of certain QC procedures that detected no deficiencies were computed. For example, it was estimated that if QC tests were applied only to new lots of selected dehydrated media and fresh batches of selected reagents, rather than to every prepared lot, costs could be reduced $2000/year. Hicock and Marshall[95] described a system for the QC of commonly used biochemicals in the clinical

laboratory that was cost-effective and convenient, and was applied to commercial identification kits as well as to single-tube media. They prepared mini-QC cultures that compared favorably with conventional tests media inoculated in parallel. Reynolds[96] proposed a nonmicrobial alternative to reagent QC, based on testing for the pure chemical form of the metabolic end products used for positive identification. The metabolite reagent control system was simple, reliable, cost effective, and eliminated the need for QC media and live microbial cultures.

Four brands of thiosulfate-citrate-bile salts-sucrose (TCBS) agar were compared for recovery of *Vibrio* species.[97] The media were prepared according to the manufacturer's instructions, and tested with inocula of *Vibrio* pure cultures, following a standardized procedure and statistical design. Tryptic soy agar with 1% NaCl served as the control. The recovery and quality of growth of each species varied significantly on the various brands of TCBS agar. A simplified statistical procedure based on the *t* test was described for laboratories routinely working with pathogenic *Vibrio* species.

Starkey[98] reviewed the development of QC biological (spore test) indicators for steam and ethylene oxide (ETO) sterilization, and attempts at standardization of these indicators. He also discussed the development of chemical indicators that produce color changes when exposed to moist heat or ETO plus moist heat. It was emphasized that biological and chemical indicators only indicate that the sterilization process has operated as planned, not that the materials are completely sterile.

Friedemann[99] reviewed the culture media used to test for sterility in various pharamacopoeias and international recommendations. A comparison and evaluation of media used for testing sterility was discussed.

The black threads on glass tube type sterilization indicators used to ensure sterility in hospitals and research laboratories were found by Atherhold *et al.*[100] to be mutagenic. Therefore, fluids autoclaved with these indicators may alter the results in mutagenicity and other studies.

Flemming[101] warned that ion exchange resins are sources of bacterial contamination, and bacterial cells can easily be washed off resin surfaces. Means of minimizing the generation of bacteria in an ion exchange bed included continuous operation, as far as possible, and keeping the resin in the regeneration agent when not in operation. Closed loop operation, back-flushing, and regeneration were insufficient in reducing bacterial growth, and could not replace disinfection of the resin.

Replicate counting errors by analysts and bacterial colony counters were investigated.[102] Errors were computed when a plate of a pasteurized milk sample was counted twice by one analyst and twice by two analysts. The results were used to make recommendations for revising standard methods for bacterial counts of milk, and to evaluate the counting accuracy of four bacterial colony counters.

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